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A microscopic view on the renal endothelial glycocalyx

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Introduction

Endothelial cells perform key homeostatic functions such as regulating blood flow and permeability, preventing leukocyte activation, and aiding immune surveillance for pathogens. Endothelial activation therefore has been identified as an important effector mechanism in progression of renal disease as well as the associated development of cardiovascular disease. The primary interface between blood and the endothelium is the glycocalyx. This carbohydrate-rich gel-like structure mediates most of the regulatory functions of the endothelium. Because the endothelial glycocalyx is a highly dynamic and fragile structure ex-vivo, studying its dimensions and function has been proven to be a challenge. Tissue processing for staining and perfusion-fixation, usually will result in a partial or complete loss of the glycocalyx. Consequently, its functions and its potential as a therapeutic target have often been underappreciated. Here we will outline the different techniques to visualize structure function relationships in kidney and vasculature.

Imaging the endothelial glycocalyx

Endothelial activation has been shown to be a central mediator in the development of cardiovascular and kidney disease [1,2]. Because the endothelial glycocalyx is the primary interface for interaction of blood with the vessel wall, it can be postulated that endothelial activation in fact is primarily mediated through changes in EG function or composition. Well known risk factors for the development for cardiovascular and renal disease such as diabetes have been associated with both upregulation of heparanase and hyaluronidase [3,4], resulting in degradation or loss of glycocalyx. For example, increased activity of heparanase has been demonstrated in both circulating mononuclear cells as well as the plasma of hemodialysis patients and linked to the presence of atherosclerotic lesions [5]. Because the EG is a highly dynamic and fragile structure, it is unstable when taken out of its in vivo environment. Consequently, bound plasma components and GAGs can be lost during fixation, dehydration, sectioning and staining procedures. As a result, only membrane bound parts of the glycocalyx will remain. Avoiding this loss of the EG is one of the biggest challenges in EG visualization. Attempts to visualize changes in EG composition and thickness, have led to imaging techniques based on different characteristics of the EG.

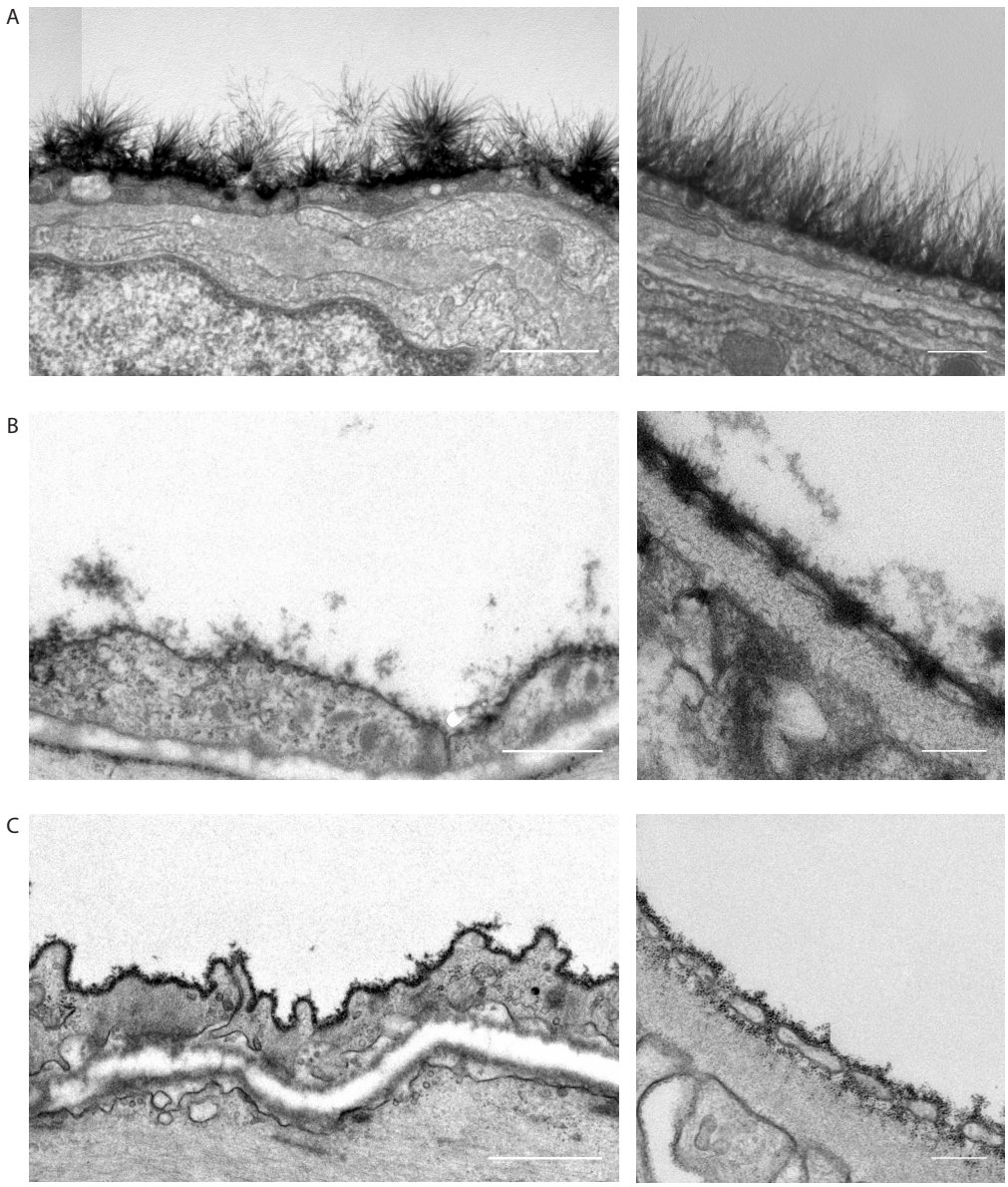

Figure 1: Three methods to stabilize and stain the endothelial glycocalyx for electron microscopy. *A) Alcian Blue 8X stained artery (left) and detailed image of an Alcian Blue 8x stained myocardial capillary. B) Cupromeronic Blue stained vein (left) and detailed image of glomerular capillary (right). C) Cationic Ferritin stained vein (left) and detailed image of glomerular capillary (right). Scale bars represent 500 nm (left) and 200 nm (right)*

Electron microscopy

Preserving and staining the EG for EM

The high polysaccharide content of the EG interacts poorly with the commonly used post-fixation stains. As a result, the EG scatters few electrons and is indistinguishable from its environment in conventionally processed samples for TEM [6]. Thus, even if the pre-treatment of the sample maintains the structural integrity of the endothelial glycocalyx, an additional staining or preservation is required for visualization of the EG in TEM. This explains the absence of visible EG structures in almost all tissues that are conventionally processed for TEM. The first to succeed in staining the EG for electron microscopy was Luft in 1966, who used ruthenium red, or ammoniated ruthenium oxychloride, which stains the aldehyde fixed mucopolysaccharides in the EG and generates an electron dense stain in the presence of osmium tetroxide [7].

The presence of the luminal EG has subsequently been shown using TEM in combination with a variety of staining and preservation techniques such as ruthenium red, ferritin, and lanthanum. Most of these agents cationic dyes bind to the negative charge of the sulfated GAGs and form an electron dense contrast together with osmium tetroxide, to enable visualization [8-12]. Using alcian blue 8GX, van den Berg et al. were the first to be able to better stabilize the anionic carbohydrate structures in myocardial capillaries, thereby visualizing an impressive EG of up to 500 nm thick [13]. The saccharine nature of these visualized structures was confirmed by perfusion of gold-labelled lectins before the staining procedure. Recently, we were able to visualize the EG in the renal glomerulus using cupromeronic blue, a chemically more stable cationic dye resembling some alcian blue properties [14]. This revealed a staining of matrix polysaccharides on the luminal surface of the glomerular endothelial membranes and a dense staining of polysaccharide matrix within the fenestrae. Examples of the described staining procedures are shown in **figure 1**.

High pressure freezing

Because optimal preservation of the EG and its visualization requires the use of perfusion fixation techniques, it has not been possible to reliably image the EG in patient biopsy material. Although not yet used for this purpose, a combination of rapid freezing and freeze substitution of the tissue might be an interesting way to preserve the EG. With a combination of instantaneous high pressure and a rapid decrease in temperature, high-pressure freezing preserves the tissue while reducing the formation of ice crystals. In a frozen state water can be replaced and tissue can be stained at the same time. This way molecules can be fixated in plastic, while preserving their native structure as much as possible. Results of this procedure are shown in the example of a high pressure frozen mouse renal sample which is stained during freeze substitution with acridine orange and uranyl (**figure 2**). Although further optimization is necessary, it does appear to be a promising technique that might pave the way for studying changes in the local EG in patients.

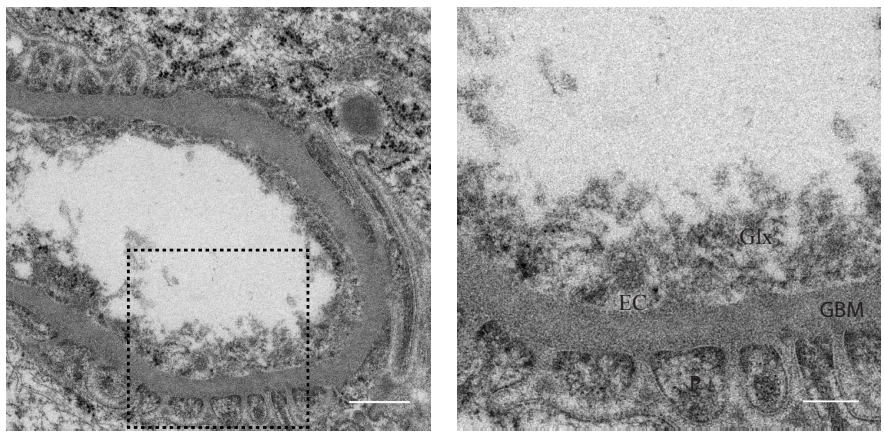

Figure 2: EG in a glomerular capillary of a high pressure frozen kidney section. *EG was stained with acridin orange and uranyl during the freeze substitution stage. Overview (left) and detailed image of the glycocalyx on top of the glomerular filtration barrier (right). EC: endothelial cell, GBM: glomerular basemenent membrane, P: Podocytes, Glx: Glycocalyx. Scale bars represent 500 nm (left) and 200 nm (right).*

3-Dimensional EM imaging

Over the recent years some interesting developments took the field of electron microscopy to a next level. These new methods might be used to better and more reliably study the EG. In regular EM imaging techniques, the highly detailed 2-dimensional EM images are prone to selection bias, as only a small area of interest is shown. To avoid this operator introduced selection bias, Faas et al. developed a method which provides high resolution and high detailed images while maintaining the lower resolution overview of the cellular context [15]. Examples of images in which virtual zooming was used on a stitch of a Cupromeronic blue stained glomerulus are shown in **figure 3**.

Interestingly, within one stitch of a mouse glomerulus different structural organizations of the EG can be observed, depending on its location (figure 3B non-fenestrated (top) vs fenestrated (bottom) endothelium). In addition 3D imaging can also be used to decrease bias and obtain more insight in the imaged structures. Based on earlier publications where bacterial flagellae were imaged [16], Arkill et al. succeeded to make a 3D reconstruction of the EG using 3D electron tomography. In electron tomography, an electron beam passes through the sample which is tilted after every image so that images can be taken at different angles. By reconstructing these images, a 3D image of the sample can be produced [17]. Although not used for EG imaging yet, some newer techniques, like SEM with a built-in microtome and focused ion beam scanning electron microscopy (FIB-SEM), will make the 3D analysis in electron microscopy even more detailed [18].

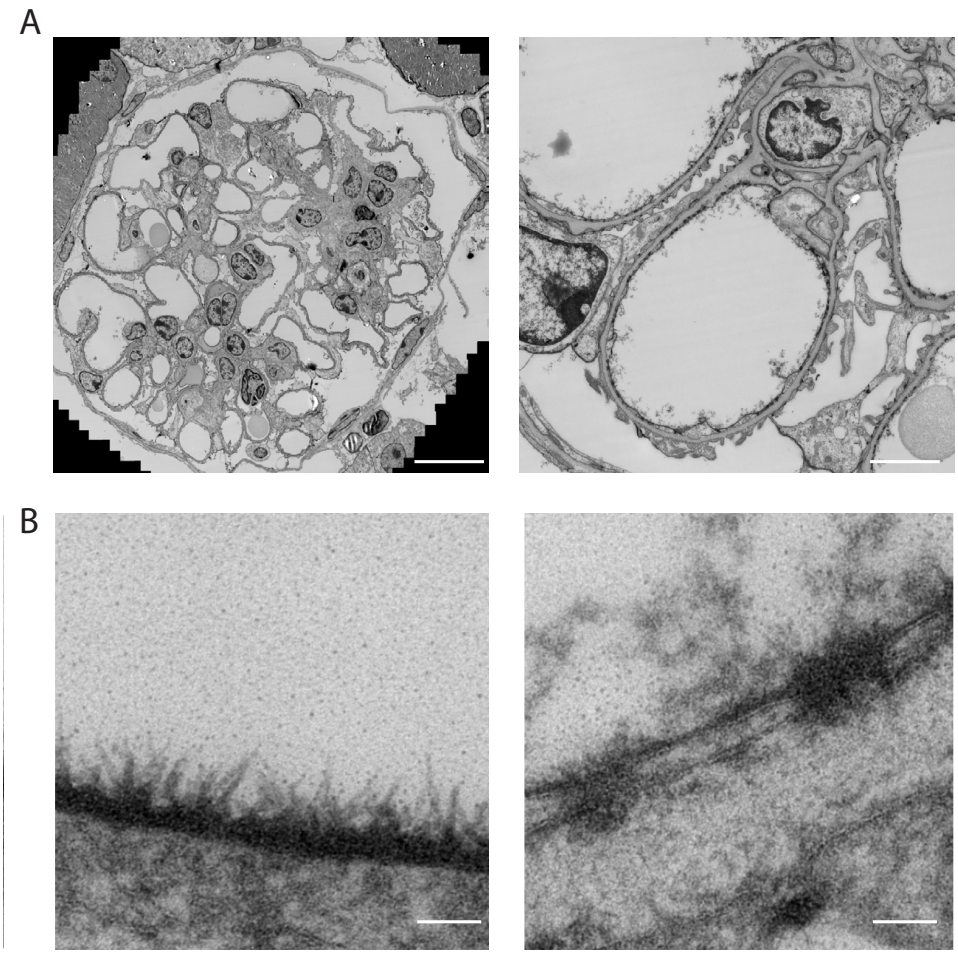

Figure 3: Transmission electron microscopic images of a cupromeronic blue stained glomerulus. *A) Overview (left) and virtual zoom (right) of a stitch of a mouse glomerulus. B) Virtual zoom with maximal detail showing different structures of the endothelial glycocalyx on top of continuous endothelium (left) and fenestrated endothelium (right). Scale bars represent 10 µm (left) and 1 µm (right) (A) and 100 nm (B).*

Fluorescence microscopy

Staining and imaging specific EG components

The carbohydrates within the EG have been imaged using specific lectins, mostly in combination with fluorescence microscopy. Lectins are carbohydrate binding proteins that recognize specific sugar moieties and 3D configurations. One of the most commonly used lectins is wheat germ agglutinin (WGA). Although lectin staining of the EG can be used to study changes in dimensions, they do not allow to determine changes in the specific composition of different GAGs. Consequently, antibodies are needed to more specifically determine compositional changes within the EG.

Over the recent years several antibodies have been obtained against specific carbohydrate domains within heparan sulfates (e.g. JM403, 10E4 and HEPSS1), hyaluronan and chondroitin sulfates or against the proteoglycan core proteins such as syndecan, glypican or perlecan [19-24]. Chondroitin sulfate and hyaluronan have been shown to be present on the endothelium, using an anti-CS antibody and by capitalizing on the protein binding properties of HA with a fluorescent hyaluronan binding protein (HABP), respectively [24-26]. Although all these techniques have shown the presence of glycocalyx components on the endothelial cells, a considerable part of these studies have been performed using epi-fluorescence microscopy, which has major limitations with respect to optical spatial resolution necessary for imaging the luminal endothelial glycocalyx.

With the development of confocal and two photon laser scanning microscopy (TPLSM), fluorescence microscopy has been improved in such a way that the resolution is high enough to be used for detailed EG imaging in situ. Examples of in situ stained mouse glomerulus and WGA perfused isolated carotid arteries, imaged with multiphoton microscopy are shown in **figure 4**. The so-called super-resolution microscopy, such as stimulation emission depletion (STED), ground state depletion (GSD) and saturated structured illumination microscopy (SSIM) even enables higher detailed imaging than the conventional confocal microscopy.

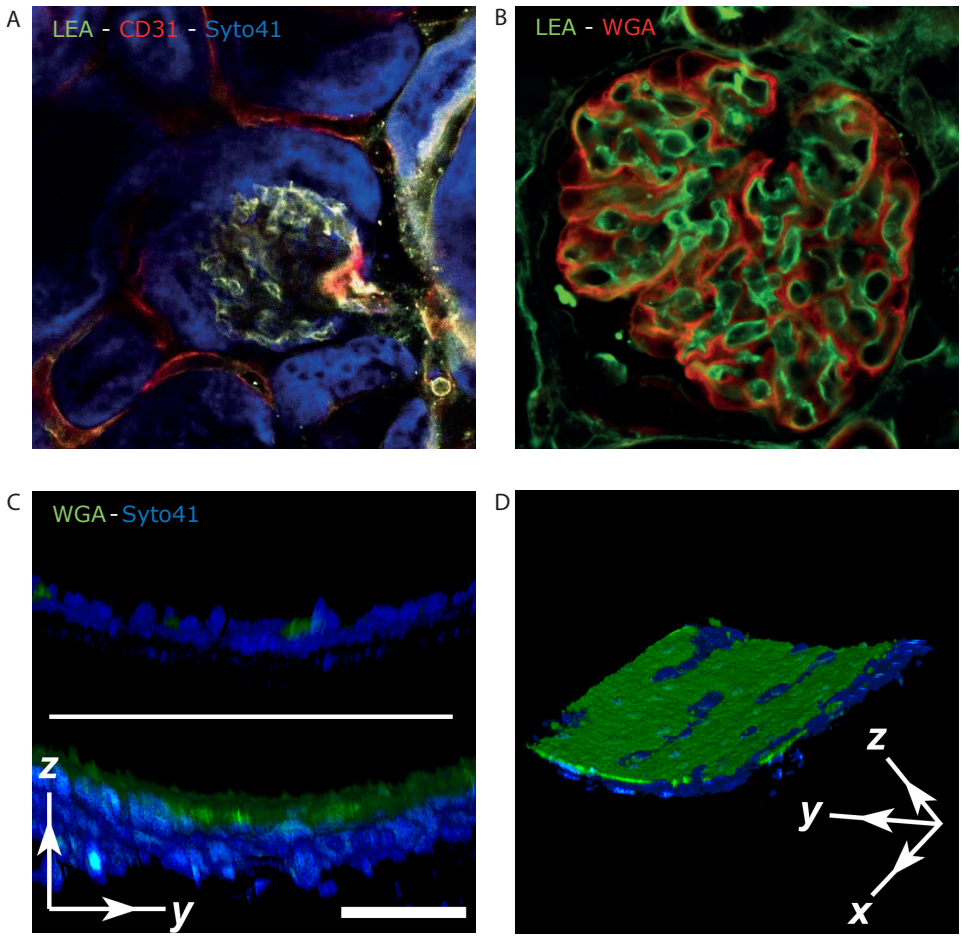

Figure 4: Endothelial glycocalyx imaged ex vivo with confocal- or multiphoton imaging techniques. *A) Mouse glomerulus visualised with anti-CD31 antibodies and TRITC-labeled conjugate for endothelium (red), syto41 for nuclei (blue) and lycopersicon esculentum (LEA) for EG (green).B) Mouse glomerulus visualised with LEA (green) and wheat-germ agglutinin WGA (Red). C-D) Mouse common carotid artery images obtained with two-photon laser scanning microscopy showing part of the vessel wall. Vessel was perfused with FITC-labeled WGA (green) and SYTO 41 (blue). Endothelial glycocalyx is almost inaccessible to WGA in normal situations (C, upper part) and becomes visible after exposure to a small air bubble (C, lower part). 3D reconstruction shows that the endothelial glycocalyx stained by WGA covers the endothelium (D) Bar represents 50 µm. The arrows indicate the direction of the X, Y, and Z axis.*

New labels for imaging the EG: Single chain antibodies

A complex but promising field for studying heparan sulfates within the EG is the use of single chain antibodies. For the selection of these single chain antibodies, a synthetic single-chain variable fragment library is used. Out of these single chain variable fragments, specific antibodies can be selected. In this way, antibodies against tissue specific modified HS domains can be produced. This method has been used previously to study heparan sulfate domains present in the basement membrane [27]. Although the same principle is used to study the role of heparan sulfates present on glomerular endothelial cells [28], it has never been used to image these heparan sulfates specifically on the luminal endothelial surface. Therefore, the use of single chain antibodies in conditions that allow for EG staining might be interesting to obtain more insight in the complexity of the different subcomponents within the EG. This promises to be an interesting field for the development of new antibodies specific for certain GAG domains within the luminal EG.

Measuring and imaging structural characteristics

Circulating markers of the glycocalyx

In response to reactive oxygen species (ROS) or other inflammatory mediators, both single GAGs and proteoglycans can be shed from the endothelium [29-31]. This has been suggested to be mediated by endoglycosidases such as heparanase and hyaluronidase, or proteases such as matrix metalloproteases [30,32,33]. Consequently, measuring shed glycocalyx components might be used as a marker for endothelial glycocalyx stability. For example, syndecan-1 and heparan sulfate are released from the tissue and can be detected in the circulating blood of patients with perioperative global or regional ischemia [34,35]. The glycocalyx core protein syndecan-1 acts as negative regulator of endothelial activation [36] and has been shown to be critical for processing and inactivation of heparanase on the cell surface [37]. In addition, release of the endothelium specific proteoglycan thrombomodulin has been shown to coincide with diabetes and diabetic nephropathy [38,39]. We demonstrated that patients with renal failure have increased circulating levels of syndecan-1 and thrombomodulin, which was reversed by kidney transplantation [40]. Furthermore, renal failure was shown to correlate with increased concentrations of shed HA [41]. Altogether, while several glycocalyx components are shedded upon endothelial activation, the exact location, mechanism and timing of shedding is complex and mostly unknown. Interpreting the results is still challenging.

Atomic Force microscopy

To determine the elastic properties of the EG, atomic force microscopy (AFM), has been shown to be a valuable tool. An AFM consists of a cantilever with a spherical tip which can scan the surface of the specimen. Reaching close contact with the specimen, forces between the tip and sample will result in deflection of this cantilever, that can be detected by changes in reflection of a laser spot. This technique has been previously used to measure mechanical stiffness in endothelial cells. However, by comparing endothelial cell layers with and without removal of the EG, the presence and stiffness of the EG could also be measured. This resulted in an estimated thickness of 400 nm of the EG on cultured endothelial cells, with a 50% EG thickness reduction after heparinase treatment [42]. Changes in the endothelial surface layer stiffness and thickness were associated with both endothelial activation and shedding of syndecan-1 and HA in renal failure patients and in animals [43].

Exclusion of macromolecules

Using intravital microscopy, a luminal microdomain that is inaccessible for erythrocytes, leukocytes and macromolecules (e.g. 70 kDa dextran, dex70) can be demonstrated. For small molecules however, this microdomain is still accessible. When damaging this layer, using a 1-2 minute light-dye treatment, erythrocytes and dex70 are able to enter this microdomain (**figure 5A,B**) [44]. In addition to the demonstration of size exclusion, this technique can also be used to show the charge exclusion properties of the EG (**figure 5C**) [45].

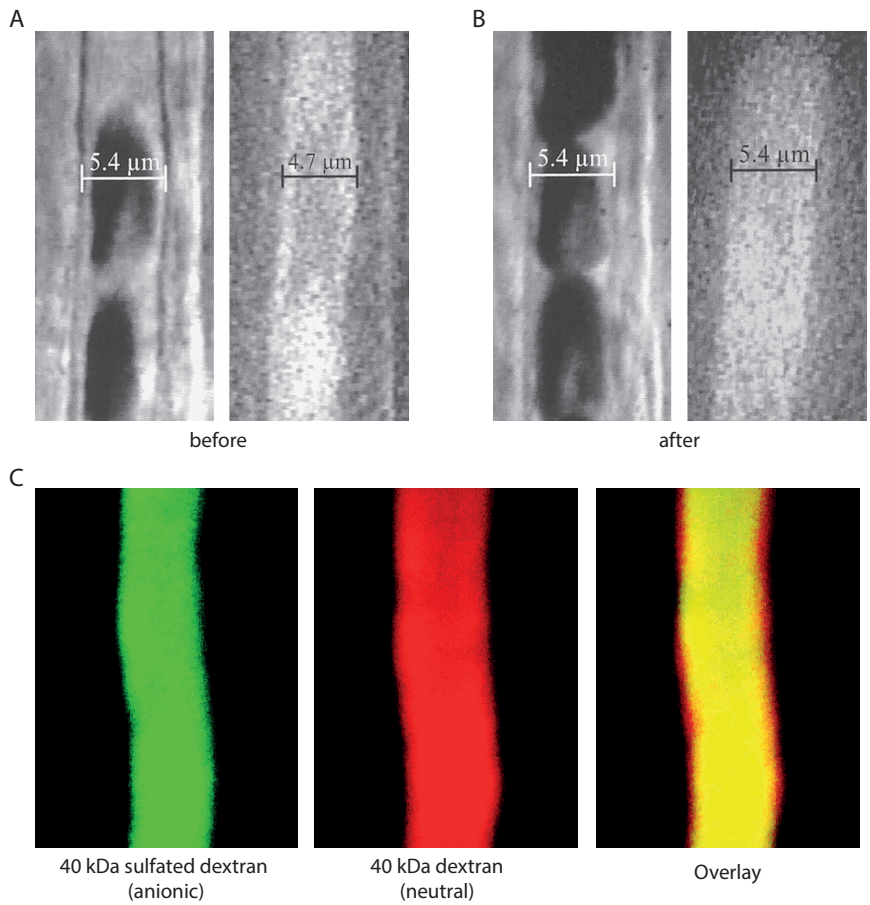

Figure 5: Exclusion properties of the endothelial surface layer in vivo. *A) Intravital microscopic image a hamster cremaster muscle capillary. The anatomical diameter of 5.4 µm is clearly larger than the red blood cell column width (left pane) or the plasma column width (right pane) labelled with fluorescent dextran (70 kD). This is caused by the endothelial glycocalyx (EG), as it is impermeable for Dex70 and RBCs. B) Image of the same hamster cremaster muscle capillary after light dye treatment (for methodology see ref [44]). The anatomical diameter of 5.4 µm is the same as the red blood cell column width (left pane) or the plasma column width (right pane) labelled with fluorescent dextran (70 kD), suggesting a destruction of the EG. C) Anionic sulfated 40 kDa dextran (green, left) and neutral 40 kDa dextran (red, middle) in mouse cremaster tissue capillaries. Difference in distribution within the same capillary (overlay, right), shows the charge-based exclusion of the anionic dextran from the endothelial surface layer.*

Most of the techniques developed to estimate the changes in EG are based on this principle. Haraldsson et al. used Intralipid, a chylomicron-like suspension of purified soybean oil, egg-yolk phospholipids, glycerol, and water to use the physical exclusion properties of the EG for determining the EG thickness in TEM. After making electron micrographs of capillaries, the location of the lipid drop was identified as central or peripheral (being within 200 nm from the luminal endothelial surface) [46]. By comparing the distribution of these lipid particles before and after for example adriamycin treatment, changes in EG thickness could be estimated [47]. In vivo, a theoretically comparable method was used by Smith et al., who looked into the location of flowing microspheres. Using dual-flash epi-illumination, the velocity of infused fluorescently labelled microspheres was estimated in the near wall plasma-rich region of venules before and after light-dye treatment to degrade the EG. Using this technique, the theoretically suggested hydraulic resistivity of the surface layer was demonstrated [48]. Also, the EG thickness was estimated to be 0.33-0.44 um, which confirms the measurements by Vink and Duling [44].

Tracer dilution technique

Based on their in vivo observations in mice, the group of Vink et al. has mainly focused on the development of a method to measure changes in EG in patients. The tracer dilution technique was the first technique to estimate the glycocalyx volume. By comparing the circulating blood volume, using a glycocalyx impermeable tracer, and the total intravascular volume, using a glycocalyx permeable tracer, the total glycocalyx volume could be estimated. This concept was first applied when Nieuwdorp et al. showed that both acute hyperglycemia and type 1 diabetes mellitus coincided with a reduction of the estimated EG volume [49,50]. Unfortunately this technique has the disadvantage that it is very time-consuming and invasive to infuse labelled RBCs and Dex40.

RBC-EC gap and SDF imaging

Based on previous observations of the RBC exclusion zone in animal models, the presence of an RBC exclusion zone between the RBC column and the endothelium was demonstrated in trans-illuminated hamster cremaster muscle capillaries [51,52]. More recently we tested the hypothesis that the EG occupies this RBC-EC gap. When comparing the width of the RBC column with the internal anatomical vessel diameter as defined by the position of the fluorescent endothelium a decreased RBC-EC gap was observed after degradation of the EG with hyaluronidase [41].

In addition, Han et al. demonstrated a decrease in this RBC-EC gap when a leukocyte was passing through the capillary. Based on earlier data and a rapid recovery of the RBC-EC gap, it was concluded that the passing leukocyte temporarily compresses the EG [53]. Consequently, the local EG thickness can be estimated in vivo by measuring the difference of the RBC column width before and directly after the passing of a leukocyte. This hypothesis was validated when the EG was degraded by oxidized LDL after which no change in RBC-EC gap could be observed before and after leukocyte passage [54].

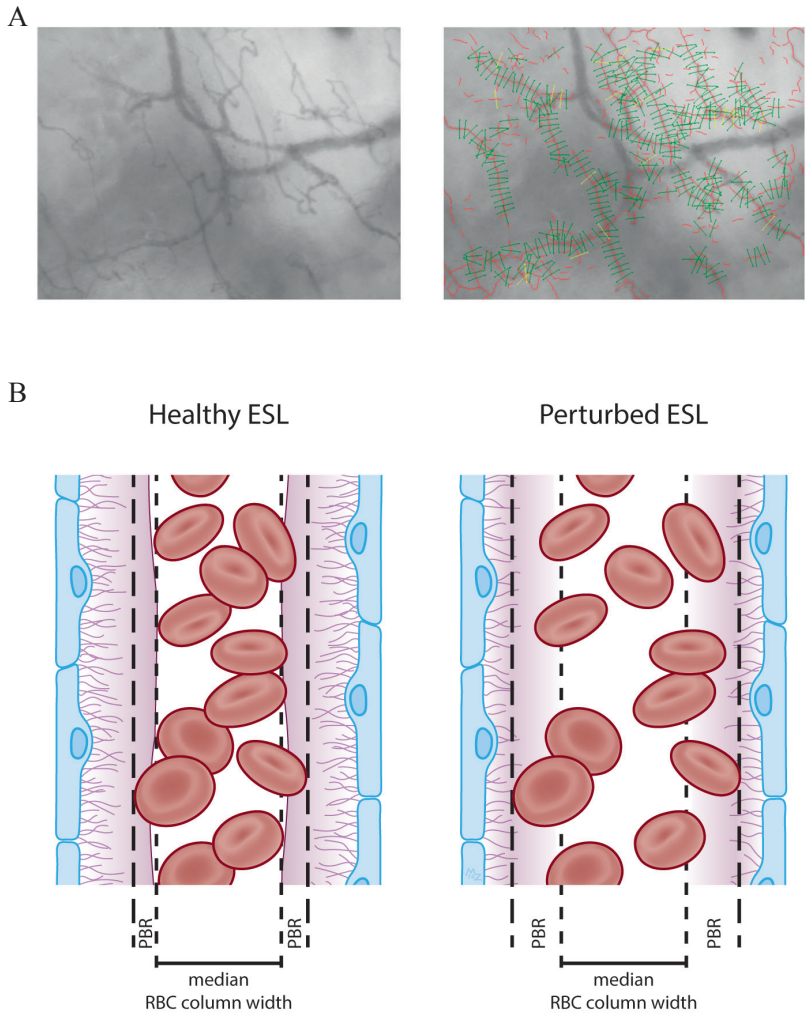

Figure 6: Sidestream darkfield (SDF) imaging to measure the perfused boundary region (PBR) in the sublingual capillary bed. *A) Recordings from the sublingual capillary bed made with the SDF camera (left). Capillaries are automatically recognized and analysed after various quality checks (right). Based on the shift in RBC column width in time, the PBR can be calculated. B) Model of a blood vessel showing the PBR in a healthy situation (left) the endothelial glycocalyx prevents the RBC to approach the endothelial cell, thus a small PBR is measured. In a disease situation (right) (40, 55, 57, 60), or after enzymatical endothelial glycocalyx (EG) breakdown in an animal-model,(41) the damaged EG allows the RBCs to approach the endothelium more often. This results in a higher variation in RBC column width reflected by a high PBR. ESL: endothelial surface layer/ glycocalyx.*

Using side stream darkfield (SDF) imaging the ability of the RBC to access the EG in the microcirculation can be measured. The technique uses light with a wavelength within the absorption spectrum of hemoglobin. A connected video camera visualizes the scattered light from the illuminated tissue [55]. Using this wavelength, the RBC column of the superficial microvasculature can be imaged in vivo. Based on the concept that an RBC can penetrate deeper into an instable or damaged EG, variations in RBC column width in time can be measured and taken as a proxy for EG accessibility. The intraluminal RBC perfused region that is situated in between the median RBC column width and unperfused domain of glycocalyx, has been coined the perfused boundary region (PBR) (**figure 6**). Deeper penetration of RBCs into the compromised glycocalyx results in increased dimension of PBR. We and others have used this technique to determine changes in EG in patients compared to control participants. In these studies, the PBR was demonstrated to be associated with microvascular perfusion and to be increased in patients with coronary artery disease, sepsis and renal failure [40,56-58]. In addition, PBR was also associated with microvascular changes in the retinal vascular bed [59]. A recent study, however, failed to demonstrate a distinction between the PBR of a pooled group of participants with a variable increased cardiovascular risk profile, stressing the need for a better understanding of PBR profiles in different patient subclasses based on gender and e.g. ethnic background [60]. Nonetheless, patient studies and the validation studies in animal models suggest that measuring the PBR to estimate microvascular risk is a promising concept.

Summary and conclusion

The endothelial glycocalyx is proposed to mediate almost all of the endothelial functions and structural or compositional perturbation of this glycocalyx associates with a variety of vasculature related pathologies. However, monitoring the EG is challenging, since the stability of the layer highly depends on its environment (e.g. blood pressure, loosely bound plasma proteins and water). Nevertheless, several imaging methods have been developed to study the endothelial glycocalyx over the last decades. Using electron microscopy, the presence of the endothelial glycocalyx has been observed in vascular tissue. An important remaining future challenge for imaging with electron microscopy (but also for other visualization techniques) is the preservation of the EG without using stabilizing perfusion fixation techniques. This would allow staining and dimensional quantification of the EG in conventionally processed patient biopsies.

To study compositional changes in the glycocalyx, at least 3D imaging should be used. Nonetheless, to study the exact (compositional) changes within the endothelial glycocalyx in more detail, future development of new techniques to analyse GAG sequence and modifications is of crucial importance. For patient studies, non- invasively measuring changes in the EG using SDF imaging is an interesting new field. With the current techniques changes in EG in severely diseased patients can be detected noninvasively. However, further validation and optimization of this technique in healthy and diseased situations is needed to clear the way for measuring changes in EG as a risk factor for the development of vascular diseases

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